Critical role of dipeptidyl peptidase I in neutrophil recruitment during the development of experimental abdominal aortic aneurysms

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Dipeptidyl peptidase I (DPPI) is a lysosomal cysteine protease critical for the activation of granule-associated serine proteases, including neutrophil elastase, cathepsin G, and proteinase 3. DPPI and granule-associated serine proteases have been shown to play a key role in regulating neutrophil recruitment at sites of inflammation. It has recently been suggested that neutrophils and neutrophil-associated proteases may also be important in the development and progression of abdominal aortic aneurysms (AAAs), a common vascular disease associated with chronic inflammation and destructive remodeling of aortic wall connective tissue. Here we show that mice with a loss-of-function mutation in DPPI are resistant to the development of elastase-induced experimental AAAs. This is in part because of diminished recruitment of neutrophils to the elastase-injured aortic wall and impaired local production of CXC-chemokine ligand (CXCL) 2. Furthermore, adoptive transfer of wild-type neutrophils is sufficient to restore susceptibility to AAAs in DPPI-deficient mice, as well as aortic wall expression of CXCL2. In addition, *in vivo* blockade of CXCL2 by using neutralizing antibodies directed against its cognate receptor leads to a significant reduction in aortic dilatation. These findings suggest that DPPI and/or granule-associated serine proteases are necessary for neutrophil recruitment into the diseased aorta and that these proteases act to amplify vascular wall inflammation that leads to AAAs.

cardiovascular | inflammation | innate immunity | proteases

Aortic aneurysms occur in 5–9% of the population over the age of 65, and ruptured abdominal aortic aneurysms (AAAs) cause at least 15,000 deaths each year (1). Although most AAAs are small and asymptomatic, they typically enlarge over time, and ≈60% eventually require surgical repair (2). At present, there are no available therapies known to alter the progressive growth of small AAAs.

Human AAA tissues are characterized by chronic aortic wall inflammation and destructive connective tissue remodeling, including depletion of aortic elastin and fragmentation of medial elastic fibers (1). These features are recapitulated by elastase-induced AAAs in rats and mice, an experimental model widely used to investigate pathologic mechanisms contributing to aneurysmal degeneration (3–5). While abundant evidence has implicated matrix metalloproteinases in the elastin degradation that accompanies AAAs (4, 6–11), the role of serine proteases has received much less attention (12). Early studies, however, indicated that human AAA tissues express a higher amount of elastase activity compared with normal or atherosclerotic aortas and that elastase activity is highest in patients with ruptured AAAs (13–15). Immunoreactive neutrophils are also evident within the adventitia and mural thrombus of AAAs, suggesting that release of neutrophil serine proteases might play an important but poorly understood role in aneurysmal degeneration (16, 17). This notion is supported by more recent studies indicating that neutrophil depletion can suppress the development of experimental AAAs (18, 19).

We recently found that elastase-induced AAAs in the mouse are accompanied by increased aortic wall expression of dipeptidyl peptidase I (DPPI) (20), a lysosomal protease required for posttranslational processing of serine proteases contained within azurophil granules (21). These serine proteases share a high degree of homology at the cDNA and amino acid levels, and all are synthesized as preproenzymes requiring activation by DPPI granules. Indeed, mice with a loss-of-function mutation in DPPI are systematically deficient in neutrophil elastase, cathepsin G, and proteinase 3 activities, as well as cytotoxic T cell granzymes and mast cell chymase (21–23). DPPI$^{-/-}$ mice thereby provide a powerful tool to investigate the role of granule-associated serine proteases in murine models of inflammatory diseases.

Results and Discussion

DPPI-Deficient Mice Are Resistant to Elastase-Induced AAAs. To address whether DPPI might play a functional role in aneurysmal degeneration, we subjected wild-type and DPPI$^{-/-}$ mice to transient aortic perfusion with elastase and assessed the subsequent development of AAAs. There was no difference between wild-type and DPPI$^{-/-}$ mice in the extent of aortic dilatation immediately after elastase perfusion (Fig. 1*A*). After day 3, wild-type mice exhibited progressive secondary dilatation to a maximum aortic diameter (AD) of 1.37 ± 0.01 mm by day 14; in contrast, the maximum AD in DPPI$^{-/-}$ mice was only 1.06 ± 0.05 mm (Fig. 1*A*). The overall increase in AD was thereby significantly reduced in DPPI$^{-/-}$ mice (0.82 ± 0.03 mm in wild-type mice versus 0.53 ± 0.02 mm in DPPI$^{-/-}$ mice; $P < 0.0001$). Consistent with previous studies (4, 20), histologic sections of the aortic wall 14 days after elastase perfusion revealed transmural inflammatory cell infiltration and pro-

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Abbreviations: DPPI, dipeptidyl peptidase I; AAA, abdominal aortic aneurysm; CXCL, CXC-chemokine ligand; MPO, myeloperoxidase; AD, aortic diameter; CXCR, CXC receptor.

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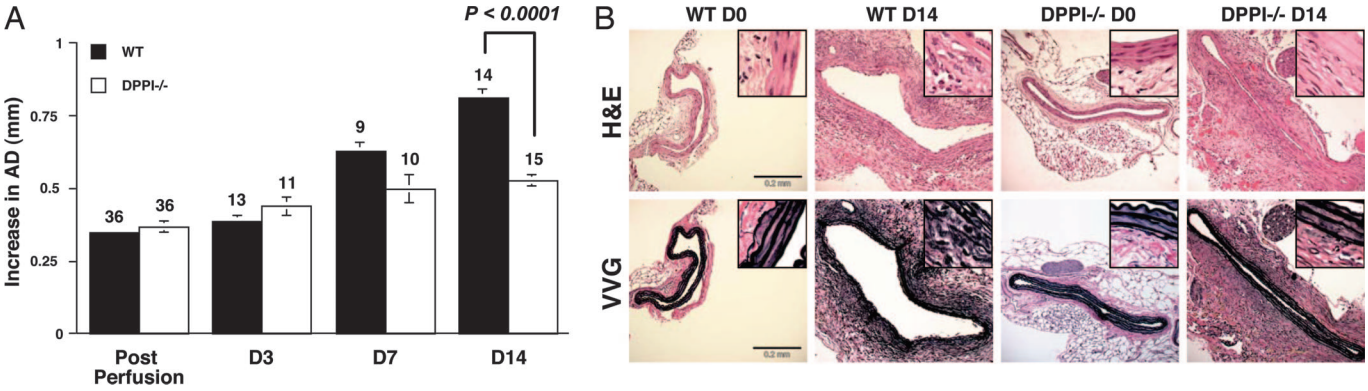

Fig. 1. DPPI is essential for the development of elastase-induced aortic aneurysms. (*A*) Groups of C57BL/6 wild-type and DPPI$^{-/-}$ mice underwent elastase perfusion and were killed at the times indicated. AD was measured for each animal and is expressed as absolute increase (in millimeters) over the baseline AD measured before elastase perfusion. In comparing wild-type versus DPPI$^{-/-}$ mice, there was no difference in the extent of initial aortic dilatation that occurred immediately after perfusion. In wild-type mice the mean AD increased by 0.82 ± 0.03 mm on day 14. In contrast, the mean increase in AD in DPPI$^{-/-}$ mice on day 14 was much lower (0.53 ± 0.02 mm). Values shown represent the mean ± SEM. The number of experimental animals per group per time point is indicated above each bar. (*B*) Histologic analysis 14 days after elastase perfusion revealed transmural infiltration by inflammatory cells and pronounced destruction of the medial elastic lamellae in wild-type animals. There was a substantial reduction in the aortic wall inflammatory response in DPPI$^{-/-}$ mice, with structural preservation of the medial elastic lamellae. (Scale bar: 0.2 mm.)

nounced destruction of the medial elastic lamellae in wild-type animals (Fig. 1*B*). The inflammatory response was substantially reduced in DPPI$^{-/-}$ mice, in association with structural preservation of the medial elastic lamellae (Fig. 1*B*). Taken together, these findings demonstrate that the extent of aneurysmal degeneration and the incidence of AAAs are both reduced in the absence of DPPI.

Neutrophils and DPPI Contribute to Aneurysmal Degeneration. We hypothesized that neutrophils and neutrophil-associated proteases are important in initiating and maintaining the inflammatory response that eventually leads to aortic degeneration. Indeed, previous studies indicate that DPPI and neutrophil serine proteases are key regulators of neutrophil recruitment at sites of inflammation and that the absence of these proteases protects mice from developing clinical arthritis (21, 24). We therefore tested whether neutrophil depletion was sufficient to protect animals from developing frank AAAs by treating wild-type mice with anti-Gr1 antibodies before elastase perfusion. Neutrophil depletion significantly attenuated the aortic dilatation observed in wild-type animals on day 14 (Fig. 2*A*). To further determine whether the resistance to AAAs in DPPI$^{-/-}$ mice was accompanied by a defect in neutrophil recruitment, we measured neutrophil influx into the aortic wall over time by different methods. Using measurements of myeloperoxidase (MPO) activity as a surrogate marker for neutrophils, we established that neutrophil influx into the aortic wall of wild-type mice reached a peak 3 days after elastase perfusion. In contrast, the level of aortic wall MPO activity on day 3 was reduced by 60% in DPPI$^{-/-}$ mice (Fig. 2*B*). This defect in neutrophil recruitment on day 3 in DPPI$^{-/-}$ mice was further confirmed by flow cytometry (Fig. 2*C*) and immunostaining for Gr1^{+} cells in the aortic wall (Fig. 2 *D–K*). Collectively, these data suggest that the protective effect against AAA development seen in DPPI$^{-/-}$ mice might be due to impaired early influx of neutrophils into the aortic wall.

If neutrophils were indeed essential for the development of AAAs, we reasoned that restoring the presence of neutrophils should cause progressive aortic dilatation in DPPI$^{-/-}$ mice. To examine this possibility, we adoptively transferred wild-type neutrophils into DPPI$^{-/-}$ mice on days 0 and 1 after elastase perfusion. This led to a significant increase in the extent of aortic dilatation in DPPI$^{-/-}$ mice on day 14 from 0.53 ± 0.02 mm to 0.75 ± 0.04 mm (Fig. 3*A*). In contrast, adoptive transfer of DPPI$^{-/-}$ neutrophils into DPPI$^{-/-}$ mice failed to cause further aortic dilatation (Fig. 3*A*), suggesting that the presence of wild-type neutrophils in the aortic wall might be necessary for AAA development. In addition, the presence of wild-type neutrophils was required early in the course of AAA development because transfer of neutrophils on day 7 failed to reconstitute the aneurysm phenotype [supporting information (SI) Fig. 5]. To evaluate whether adoptively transferred wild-type neutrophils indeed localized to the aortic wall, we used GFP-tagged neutrophils for the reconstitution. DPPI$^{-/-}$ mice were perfused with elastase followed by transfer of GFP^{+} wild-type neutrophils. Four hours after adoptive transfer, the mice were killed; the cells were isolated from the aortic wall and analyzed for the expression of GFP and Gr1 by flow cytometry. In nonreconstituted DPPI$^{-/-}$ mice, we found a small number of Gr1-expressing neutrophils in the aortic wall (529 ± 326 neutrophils per aorta) (Fig. 3*B*). In contrast, transfer of GFP^{+} wild-type neutrophils led to an increase in the absolute number of neutrophils found in the aortic wall of DPPI$^{-/-}$ mice (6,955 ± 2,305 neutrophils per aorta) (Fig. 3*B*). Of these Gr1^{+} cells, 5–11% were GFP-positive (Fig. 3*B*). These results indicate that adoptively transferred neutrophils localized to the aortic wall and suggest that these cells provided a critical signal that enabled DPPI$^{-/-}$ neutrophils to migrate effectively into the aortic wall.

DPPI-Sufficient Neutrophils Regulate Aneurysm Development Through the Production of CXC-Chemokine Ligand (CXCL) 2. Based on these above findings, we hypothesized that wild-type neutrophils recruited to the aortic wall during the initial response to elastase perfusion contributed to the generation of mediators that sustained the recruitment of leukocytes to the inflammatory site. Thus, to better understand which inflammatory mediators potentially contributed to the later phases of aneurysmal degeneration and the dynamic alterations in expression of proinflammatory cytokines and chemokines that accompanied the development of elastase-induced AAAs, we undertook gene expression studies of aortic wall tissues obtained from wild-type mice at different time intervals after elastase perfusion. Although IL-6, IL-1β, and TNF-α have all been implicated in the development of AAAs, analysis of aortic wall samples from wild-type mice showed that IL-6, IL-1β, and TNF-α mRNA did not increase significantly until day 14 (Fig. 4 *A–C*). In contrast,

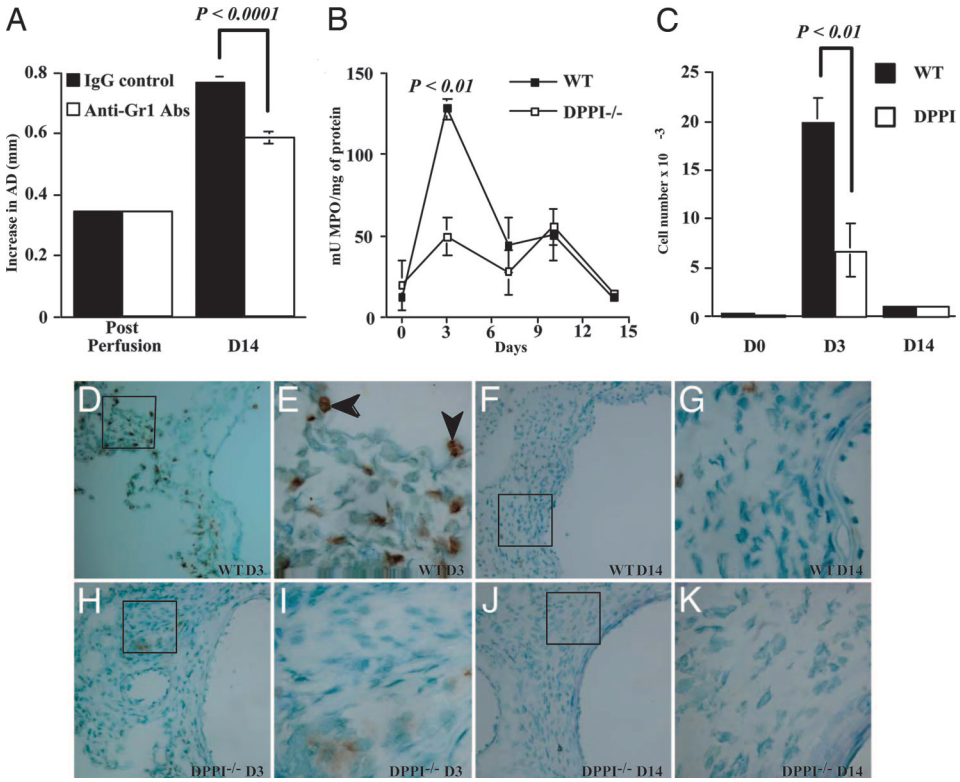

Fig. 2. Impaired neutrophil recruitment in the absence of DPPI. (*A*) *In vivo* neutrophil depletion protects against aneurysmal dilatation. Wild-type mice were injected with neutrophil-depleting anti-Gr1 antibodies or rat IgG isotype control antibodies before and after elastase perfusion. AD measurements were obtained at the indicated times. The increase in AD was significantly greater in mice treated with rat IgG (0.78 ± 0.01 mm; $n = 4$) than in mice treated with anti-Gr1 antibodies (0.59 ± 0.02 mm; $n = 6$). (*B*) By using MPO activity as a marker of neutrophils, the peak of neutrophil influx into the aortic wall was observed to occur on day 3 after elastase perfusion in wild-type animals. By comparison, the level of MPO activity on day 3 was reduced by 60% in DPPI$^{-/-}$ mice ($n = 4$ mice per genotype per time point). (*C*) Analysis of neutrophil numbers in aortic walls revealed significant differences between WT and DPPI$^{-/-}$ mice on day 3 ($20 \pm 1.8 \times 10^3$ in wild-type versus $6.8 \pm 3.9 \times 10^3$ neutrophils in DPPI$^{-/-}$ mice; $n = 3$ per genotype). The absolute number of neutrophils was calculated by multiplying the total number of cells from each aorta by the percentage of Gr1$^+$ cells. Immunohistochemistry revealed abundant brown-staining Gr1$^+$ neutrophils (arrowheads) in tissue sections obtained from wild-type mice (*D* and *E*) and confirmed a substantial reduction in aortic wall neutrophils on day 3 in DPPI$^{-/-}$ mice (*H* and *I*). The number of neutrophils was significantly decreased by day 14 in both wild-type (*F* and *G*) and DPPI$^{-/-}$ (*J* and *K*) mice. Sections indicated in *D*, *F*, *H*, and *J* (×40) are shown at higher magnification (×400) in *E*, *G*, *I*, and *K*.

aortic wall expression of CXCL2 (also known as MIP-2), a structural homologue of CXCL8 (also known as IL-8) and a potent neutrophil chemoattractant, reached peak levels in wild-type mice on day 3, increasing >15-fold over baseline (Fig. 4*D*). On the other hand, the expression of CXCL2 in the aortic tissues of DPPI$^{-/-}$ mice on day 3 remained near preperfusion levels (Fig. 4*E*). To assess whether the presence of wild-type neutrophils was sufficient to restore aortic tissue expression of CXCL2 in DPPI$^{-/-}$ mice to the levels observed in wild-type animals, we examined DPPI$^{-/-}$ mice that were perfused with elastase followed by neutrophil reconstitution. Reconstitution with wild-type neutrophils led to an increase in CXCL2 mRNA expression toward levels seen in wild-type mice, whereas reconstitution with DPPI$^{-/-}$ neutrophils did not change these levels over baseline (Fig. 4*E*). To further confirm that the neutrophil-dependent production of CXCL2 is critical for the transition to aneurysmal degeneration, we sought to block the action of this chemokine *in vivo* using neutralizing antibodies directed against its cognate receptor, CXC receptor 2 (CXCR2). Indeed, administration of anti-CXCR2 antibodies significantly attenuated the induction of aortic dilatation in DPPI$^{-/-}$ mice after adoptive transfer of wild-type neutrophils, confirming an essential role for CXCL2 in the development of AAAs (Fig. 4*F*). These results thus provide evidence that neutrophils recruited in the acute phase after elastase perfusion are directly responsible for the *in vivo* production of CXCL2, which in turn sustains the inflammatory cascade, leading to the eventual tissue destruction and aneurysmal dilatation associated with AAAs.

These experiments are the first to identify a key role for neutrophil-derived DPPI in AAAs, demonstrating that DPPI modulates the progression of aortic wall injury from an acute inflammatory response to the chronic destructive remodeling associated with aneurysmal degeneration. Because DPPI has been shown to activate neutrophil-derived neutrophil elastase, cathepsin G, and proteinase 3 (21), it remains to be determined whether the aneurysm-promoting effects are directly attributable to one or more neutrophil serine proteases. It is now recognized that neutrophil serine proteases remain active after exocytosis, and these extracellular proteases have been shown to modulate the production levels of cytokines and chemokines (25). For example, granule serine proteases can directly stimulate macrophages to release proinflammatory cytokines and chemokines (26). In addition, neutrophil elastase, cathepsin G, and proteinase 3 have all been shown to activate proteinase-activated receptor 2, leading to the production of CXCL8 and CC-chemokine ligand 2 *in vitro* (27, 28). Whether neutrophil serine proteases modulate CXCL2 level through proteinase-activated receptor 2 activation *in vivo* requires further investigation. Therefore, future studies aimed at elucidating the molecular mechanisms by which neutrophils and neutrophil-derived proteases regulate inflammatory mediator production in the aortic wall should provide novel insights and potential therapeutic strategies aimed at halting the progression of AAAs.

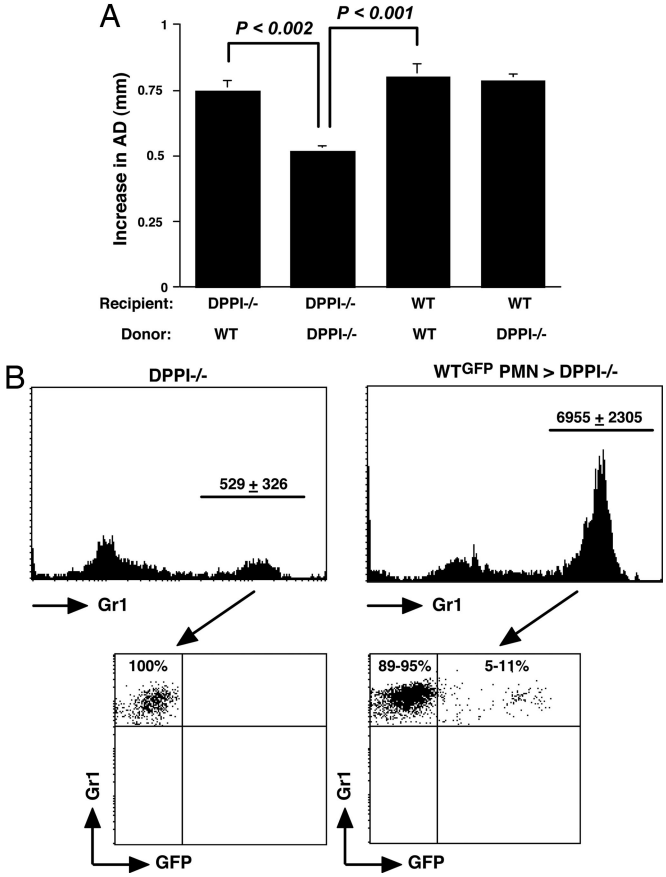

Fig. 3. Adoptive transfer of wild-type neutrophils into DPPI$^{-/-}$ mice restores susceptibility to aneurysm development. (*A*) Bone marrow-derived neutrophils were adoptively transferred into groups of mice after elastase perfusion. Transfer of wild-type neutrophils into DPPI$^{-/-}$ mice led to an increase in the extent of aortic dilatation on day 14 to 0.75 ± 0.04 mm, whereas transfer of DPPI$^{-/-}$ neutrophils into DPPI$^{-/-}$ mice failed to cause further aortic dilatation (0.52 ± 0.02 mm; $n = 4$ mice per group). Similar transfer of wild-type or DPPI$^{-/-}$ neutrophils did not change the extent of aortic dilatation on day 14 in wild-type animals (0.8 ± 0.05 mm and 0.79 ± 0.02 mm, respectively), as compared with nonreconstituted mice (0.82 ± 0.03 mm; Fig. 1*A*). (*B*) Cells were isolated from each aorta ($n = 3$–4 per group) on day 0, 4 h after elastase perfusion and adoptive transfer of GFP$^+$ wild-type neutrophils (PMN). Transfer of wild-type neutrophils into DPPI$^{-/-}$ mice led to an increase in the number of neutrophils that localized to the aortas as revealed by flow-cytometric analysis of Gr1$^+$ cells. Of these, 5–11% were GFP-positive. The absolute number of neutrophils was calculated by multiplying the total number of cells from each aorta by the percentage of Gr1$^+$ cells.

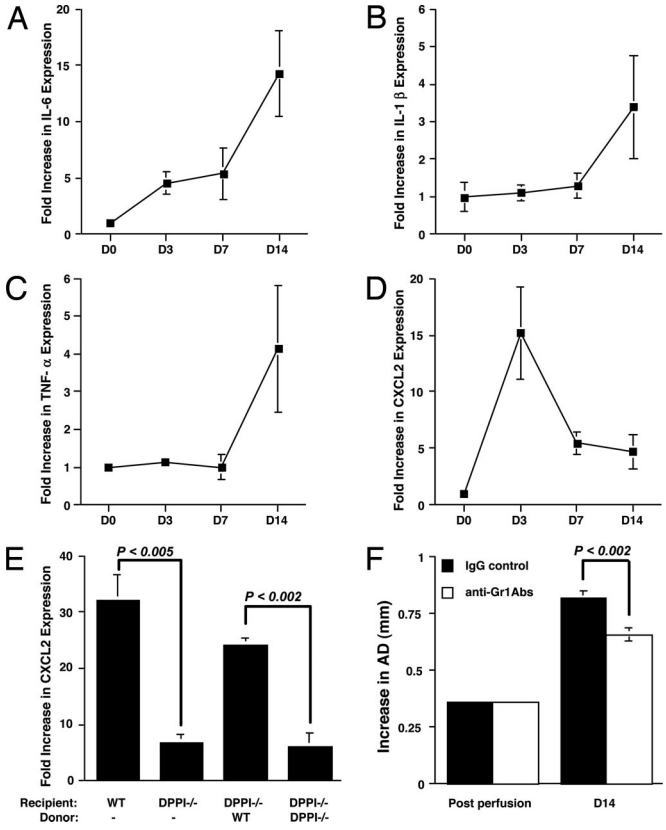

Fig. 4. Aortic wall expression of CXCL2 depends on the presence of DPPI. Groups of wild-type mice ($n = 6$ per time point) underwent elastase perfusion and were killed at the indicated time intervals. Aortic tissue expression of mRNA encoding IL-6 (*A*), IL-1β (*B*), TNF-α (*C*), and CXCL2 (*D*) was measured by quantitative real-time PCR and expressed as the fold increase over baseline values (set at 1) obtained from normal aorta (day 0). Substantial increases in IL-6, IL-1β, and TNF-α did not occur until day 14, a point when AAAs were well established. In contrast, aortic wall expression of CXCL2 reached peak levels in wild-type mice on day 3, whereas CXCL2 mRNA levels in DPPI$^{-/-}$ mice remained near preperfusion levels (*E*). Adoptive transfer of wild-type neutrophils into DPPI$^{-/-}$ mice led to an increase in the CXCL2 mRNA levels toward those observed in wild-type mice. (*F*) Administration of CXCR2-neutralizing antibodies blocked the activity of CXCL2 and attenuated the ability of adoptively transferred wild-type neutrophils to enhance aortic dilatation in DPPI$^{-/-}$ mice, whereas the injection of rat IgG isotype control antibodies did not block the action of wild-type neutrophils in DPPI$^{-/-}$ mice (increase in AD of 0.82 ± 0.03 mm in anti-CXCR2 antibody injected mice versus 0.66 ± 0.03 mm in control IgG injected mice; $n = 4$ per group).

Methods

Animals. DPPI$^{-/-}$ mice were generated in a 129/SvJ strain, as previously described (22). These animals were backcrossed to C57BL/6 mice for 11 generations by using the mutant DPPI allele as a marker for selection at each generation. Wild-type C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) along with the C57BL/6-Tg (UBC-GFP) 30 Scha/J transgenic mouse line, which systemically expresses a GFP cDNA under the control of a chicken β-actin promoter and cytomegalovirus enhancer. All animals were kept in pathogen-free conditions at Washington University Specialized Research Facility, and all experiments were performed according to protocols approved by the Division of Comparative Medicine at Washington University.

Elastase Perfusion Model of AAAs. AAAs were induced in 8- to 12-week-old male mice, as described (4). Briefly, mice were anesthetized, and a laparotomy was performed under sterile conditions with the assistance of an operating stereomicroscope (Leica, Deerfield, IL). The infrarenal abdominal aorta was isolated, and the preperfusion AD was measured with a calibrated ocular grid. Temporary 7-0 silk ligatures were placed around the proximal and distal aorta, an aortotomy was created at the bifurcation, and a microcatheter was introduced into the lumen. The isolated aortic lumen was perfused for 5 min at 100 mmHg (1 mmHg = 133 Pa) with a saline solution containing type 1 porcine pancreatic elastase (0.1455 units/ml; Sigma, St. Louis, MO). The microcatheter was then removed, the aortotomy was repaired without constriction of the lumen, and flow to the lower extremities was restored. Animals were thereafter allowed free access to food and water. At different time points, a second laparotomy was performed and the perfused segment of the abdominal aorta was reexposed and measured *in situ* before the animals were killed and tissues were procured. AD was expressed as absolute increase (in millimeters) over the baseline AD measured before elastase perfusion.

Histological Sections. Specimens of the abdominal aorta were excised after systemic perfusion–fixation with 10% neutral buffered formalin (120 mmHg for 10 min) and embedded in paraffin. Cross-sections of aortic tissue (5 μm) were stained with Verhoeff–van Gieson for elastin and with H&E and examined by light microscopy.

Immunohistochemistry. Specimens of the abdominal aorta were filled with OCT compound, excised, and snap-frozen in OCT compound. Cross-sections of aortic tissue (5 μm) were incubated with anti-Gr1 antibodies (BD Biosciences, San Diego, CA) for 1 h at room temperature, washed, and then incubated with biotinylated anti-rat IgG (DAKO, Glostrup, Denmark) for 1 h at room temperature. Color development was revealed by using the Vectastain ABC system (Vector Laboratories, Burlingame, CA). The reaction was stopped by immersing the slides in water, followed by counterstaining with 1% methyl green.

Neutrophil Depletion. Neutrophils were depleted *in vivo* by i.p. injection of 0.25 mg of rat anti-mouse Gr1 antibodies (RB6-8C5; a generous gift from Paul Allen, Washington University), administrated 48 h before the elastase perfusion procedure and 24 and 72 h later. The same dose of rat IgG_{2b} (BD Biosciences) served as control. Neutrophil depletion was confirmed by flow cytometry of peripheral blood.

MPO Assay. For quantification of neutrophil accumulation in the aortic wall, aortic tissue samples were harvested at the time points indicated, and MPO was extracted by homogenizing the minced tissues in buffer containing 0.1 M Tris (pH 7.6), 0.15 M NaCl, and 0.5% hexadecyl trimethyl ammonium bromide. MPO activity in the cleared supernatant was measured by the change in optical density at 450 nm, resulting from the decomposition of H_2O_2 in the presence of *o*-dianisidine. Purified MPO (Sigma) was used as a standard. MPO content was expressed as milliunits per milligram of protein of aortic wall tissue extracts. Protein concentrations were determined by using the BCA protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

FACS Analysis. Mice were killed on the indicated days, and the aortas were excised from below the infrarenal arteries to just above the bifurcation. The blood content was flushed, and the aortas were minced finely and shaken in 1 ml of RPMI medium 1640 supplemented with 10% FCS, 62.5 units/ml collagenase VIII (Sigma), and 0.625 units/ml Dispase (BD Biosciences) at 37°C for 1 h. The cells were passed through a 70-μm cell strainer to remove debris, counted, stained with anti-Gr1 antibodies (BD Biosciences), and analyzed by flow cytometry.

Adoptive Transfer of Neutrophils. Neutrophils were isolated from bone marrow by a discontinuous Percoll gradient, as previously described (29). Neutrophil purity was consistently 80–90% as assessed by flow cytometry. The other cells in the preparation include a small percentage of nucleated red blood cells and B cells (SI Fig. 6). Isolated neutrophils were resuspended in PBS (1×10^7 neutrophils in 200 μl of PBS) and injected i.v. into mice on day 0 after elastase perfusion and on postperfusion day 1. In some studies, mice were also administered 0.25 mg of anti-CXCR2 antibodies (R & D Systems, Minneapolis, MN) by i.p. injection on days 0 and 1, after adoptive transfer of neutrophils. Rat IgG_{2a} antibodies served as control.

Chemokine Analysis. Aortic wall expression of IL-1β, TNF-α, IL-6, and CXCL2 mRNA was measured by quantitative real-time RT-PCR. Briefly, three sets of aortas, each derived from two mice, were harvested at specific time intervals and pulverized under liquid nitrogen. Total RNA was isolated by using TRIzol reagent (Gibco/BRL, Grand Island, NY) as recommended by the manufacturer. Two micrograms of total RNA from each sample was used to synthesize cDNA by reverse transcription on a GeneAmp 9600 thermal cycler system, according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA). The reverse transcription products served as the template for real-time PCR analysis by using reagents and protocols provided in the SYBR Green PCR kit and the GeneAmp 5700 Sequence Detection System (Applied Biosystems). The following gene-specific primers were used: IL-1β forward primer, 5′-ACTACAGGCTCCGAGATGAA-3′; IL-1β reverse primer, 3′-TGGGTCCGACAGCACGAGGC-5′; CXCL2 forward primer, 5′-CGCTGTCAATGCCTGAAG-3′; CXCL2 reverse primer, 3′-GGCGTCACACTCAAGCTCT-5′; TNF-α forward primer, 5′-CCTGGCCAACGGCATGGATC-3′; TNF-α reverse primer, 3′-CGGCTGGCACCACTCGTTGG-5′; IL-6 forward primer, 5′-CCTAGTGCGTTATGCCTAAGCA-3′; and IL-6 reverse primer 3′-CCACAGTGAGGAATGTCCACAA-5′. PCRs were performed in triplicates with SYBR Green PCR Core Reagents (Applied Biosystems), and fluorescent signals were analyzed by using GeneAmp 5700 Sequence Detection System software (version 1.3; Applied Biosystems). Results for each sample were normalized to the concentration of β-actin mRNA measured in the same samples and expressed as fold increase over baseline levels on day 0, which are set at a value of 1.

Statistics. Data are presented as the mean ± SEM. Student's *t* test was used to compare the means of two independent sample groups. For multiple-group comparison option, the Tukey–Kramer method was used. P values <0.05 were considered significant.

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